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Tissue-specific expression of glutathione peroxidase gene in guinea pigs

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Glutathione peroxidase (GSH-Px), a selenocysteine-containing enzyme, is generally considered to be important in protecting animals from oxidative injury. However, guinea pigs have very low GSH-Px activity in major tissues such as liver and kidney, while the activity in the erythrocytes is as high as that of mice or rats. The present study attempted to clarify which step in the gene expression of GSH-Px is responsible for the tissue specific regulation of GSH-Px activity in guinea pigs. Northern blot analysis showed clear signals of GSH-Px mRNA in the reticulocytes and erythroblast-enriched bone marrow cells of guinea pigs, while it was barely detectable in the liver, kidney and heart. Using the nuclear run-on assay, we confirmed that the difference in GSH-Px mRNA levels among tissues of guinea pigs results primarily from the difference in the transcription rate of the GSH-Px gene. Thus, the guinea pig may be a good model for studying the factors regulating the tissue-specific gene expression of this selenoenzyme as well as its essential role.

Introduction

Glutathione peroxidase (GSH-Px) is a selenoenzyme which catalyzes the reduction of hydrogen peroxide (H_2O_2) and organic hydroperoxides. Together with catalase and superoxide dismutase, GSH-Px is considered to play an important role in protecting animals from oxidative injury [1]. Recently, it has been demonstrated that the selenocysteine moiety, the active site of GSH-Px, is coded by UGA termination codon [2] and that the incorporation of selenocysteine into the polypeptide chain of GSH-Px involves the utilization of suppressor tRNA in a complicated manner although the precise mechanism has not been completely elucidated [3,4].

GSH-Px is generally recognized as a house-keeping enzyme with its highest activity observed in the liver or kidney in most mammals. However, several studies have revealed that guinea pigs show extremely low GSH-Px activity in the liver [5,6]. We investigated expression of the GSH-Px gene in rodents including guinea pigs and found that guinea pigs have a gene highly homologous to mouse GSH-Px in the genome, while mRNA of GSH-Px was barely detectable in the liver, kidney and heart of this species [7]. These findings suggest that the markedly low activity of GSH-Px in guinea pig tissues may be due to the low rate of transcription of the GSH-Px gene.

Recently, however, in a more detailed study on species differences in GSH-Px activity among rodents, we demonstrated that GSH-Px activity in the erythrocytes of guinea pigs is as high as that of mice or rats [8]. Therefore, this study attempted to examine the expression of the GSH-Px gene in guinea pig cells from which the erythrocytes originate. Results of Northern blot analysis showed a higher mRNA level of GSH-Px in the reticulocytes of guinea pigs than that in other tissues.

In selenium deficiency, mRNA levels of GSH-Px have been shown to decrease in the tissues of selenium-deficient rats and mice [7,9,10], suggesting a regulatory effect of selenium on the transcription step of the GSH-Px gene. However, we have demonstrated using a nuclear run-on assay that the rate of GSH-Px gene transcription in isolated nuclei obtained from selenium-deficient mice did not differ from that of selenium-sufficient mice [11]. This phenomenon was also observed in rats [12,13]. These data indicate that the level of mRNA of GSH-Px does not necessarily reflect the transcription rate of the GSH-Px gene. Therefore, in the present study, we carried out a nuclear run-on assay to clarify whether the difference

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in the level of mRNA among tissues in guinea pigs actually results from the difference in the transcription of this selenoenzyme gene.

Materials and Methods

Animals and tissue samples

5-Week-old male ICR mice (Charles River Japan, Atsugi, Japan) and Hartley guinea pigs (Japan SLC, Hamamatsu, Japan) were used. Mice were fed CE-2 (CLEA Japan, Tokyo, Japan) and guinea pigs were fed Labo-G (Nippon Nosan, Yokohama, Japan). Selenium contents of these diets were 0.42 ppm and 0.47 ppm, respectively. Blood samples were collected from the vena cava under anesthesia with a heparinized syringe. For the extraction of total RNA, 0.5 g of tissue sample freshly obtained from three animals was homogenized in 6 ml of 4 M guanidium thiocyanate buffer containing 1% mercaptoethanol, 0.5% sarkosyl and 25 mM sodium acetate (pH 6.0) [14]. The homogenates were obtained in duplicate and frozen at −80°C until RNA extraction. For the nuclear run-on assay, the nuclei were isolated from the liver tissue immediately after tissue collection by the method of Schibler et al. [15]. Residual tissue samples were frozen at −80°C for the measurement of GSH-Px activity.

Collection of reticulocytes and bone marrow cells

For the collection of reticulocyte-rich blood, guinea pigs were intraperitoneally injected with 40 mg/kg of acetyl phenylhydrazine for 5 days and killed on the third day after the last injection. Blood samples were centrifuged at 2500 rpm for 5 min at 4°C, and after the aspiration of plasma and buffy coat, 0.5 ml of red blood cells in the upper layer were taken for the extraction of total RNA. From the phenylhydrazine-treated and non-treated animals, bone marrow cells were obtained by washing out the femur with saline and centrifuging the wash-out at 2500 rpm for 5 min at 4°C. The precipitated cells were suspended in saline. A portion of the suspension was used for total RNA extraction and the residue was used for nuclei isolation.

Measurement of GSH-Px activity

The activity of GSH-Px in the cytosol of tissues was determined by the method of Lawrence and Burk [16] using H_2O_2 (0.25 mM) as a substrate. The cytosol was obtained by centrifugation of the tissue homogenates at 105 000 × *g*. For measurement of GSH-Px in the erythrocytes, *tert*-butylhydroperoxide (0.3 mM) was used instead of H_2O_2, since hemoglobin has a peroxidase-like activity with H_2O_2 [17]. The concentration of each substrate was selected so that the same amount of GSH-Px would show the same activity. Protein concentration was determined by Lowry's method [18].

Southern blot analysis

High-molecular-weight DNAs were purified from the liver of mice and guinea pigs as described previously [7]. DNAs were digested independently with several kinds of restriction enzyme, separated by electrophoresis in 0.8% agarose gels and transferred to nitrocellulose filters. Mouse β-actin cDNA was cloned from a mouse cDNA library (gift of Dr. Kuge) in our laboratory. Mouse hypoxanthine-guanine phosphoribosyltransferase (HPRT) cDNA developed by Caskey et al. [19] was obtained from JCRB (Japanese Cancer Research Resources Bank). The uniformly ^{32}P-labeled probes were generated using a random primer DNA labeling kit (Takara, Japan). The blots on the nitrocellulose filter were hybridized to mouse β-actin cDNA or mouse HPRT cDNA at 42°C for 15 h in 40% formamide, 3 × SSC, 0.1% SDS, 10 × Denhardt's solution, and 50 μg/ml of denatured salmon sperm DNA, washed in 0.15 × SSC and 0.1% SDS at 50°C for 1 h and subjected to autoradiography. 1 × SSC contained 0.15 M NaCl and 0.015 M sodium citrate, and 1 × Denhardt's solution contained 0.02% Ficoll, 0.02% poly(vinylpyrrolidone) and 0.02% bovine serum albumin.

Northern blot analysis

Total RNA was prepared from tissue homogenates of mice and guinea pigs using the guanidium thiocyanate/CsCl method [20]. Northern blot analysis of total RNA was performed in basically the same way as described previously [7] using mouse GSH-Px cDNA and mouse β-actin cDNA as probes. We have already cloned a cDNA fragment of mouse GSH-Px from mouse liver cDNA library [7], determined the nucleotide sequence by the dideoxy termination method [21] using Sequenase sequencing kits (United States Biochemical Corporation), and confirmed the sequence to be completely identical to the exon regions (nucleotides 45–283 and 500–1146) of mouse GSH-Px gene reported by Chambers et al. [2]. 40 μg total RNA per lane was loaded on the gel for electrophoresis. The concentration of RNA in the preparation was monitored spectrophotometrically (absorbance at 260 nm) and the purity of RNA was confirmed by 1% agarose gel electrophoresis after denaturation of RNA with glyoxal. Conditions for hybridization to each probe and washing of the nitrocellulose filter were the same as in Southern blot analysis.

For HPRT, poly(A)$^+$ RNA was prepared from the total RNA of the liver and bone marrow cells of guinea pigs using an oligo(dT) column. 7 μg of poly(A)$^+$ RNA was subjected to Northern blot analysis in the same way as GSH-Px and β-actin.

Nuclear run-on assay

Nuclei were isolated from the liver of mice, and the liver and bone marrow cells of guinea pigs. The isola-

tion of nuclei including RNase treatment [15] and nuclear run-on assay was performed in the same way as described previously [11]. Briefly, the isolated nuclei were incubated for 20 min at 30°C with a reaction mixture containing 5.2 MBq of [α-^{32}P]UTP (30 TBq/mmol, Amersham), 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 300 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.0) and human placental RNase inhibitor (Takara Shuzo, Japan). After termination of the reaction by the addition of deoxyribonuclease I, the RNA transcribed was obtained by means of TCA precipitation. The labeled transcripts were hybridized to slot blots of mouse GSH-Px cDNA, β-actin cDNA or HPRT cDNA. The plasmid vector pUC118, into which each cDNA was cloned, was used as a negative control. The blots were hybridized at 42°C for 48 h and washed and subjected to autoradiography following the same procedure as the Northern blot analysis described above. Signals in the autoradiogram were quantitated by scanning with a spectrodensitometer (PhastSystem, Pharmacia).

Results

Fig. 1 shows the GSH-Px activity in the liver, kidney, heart, lung, testis, brain and erythrocytes of mice and guinea pigs. Guinea pigs showed an extremely low activity of GSH-Px in the liver, kidney and heart compared with that in mice. In the erythrocytes, however, the activity of GSH-Px in guinea pigs was comparable to that in mice and was higher than in any other tissues of guinea pigs.

To elucidate the step in gene expression responsible for the marked difference in hepatic GSH-Px activity between mice and guinea pigs, Northern blot analysis and the nuclear run-on assay were performed. Gene expression of β-actin in these species was used as a control. To examine the cross-hybridization of mouse β-actin cDNA to guinea pig gene, we performed

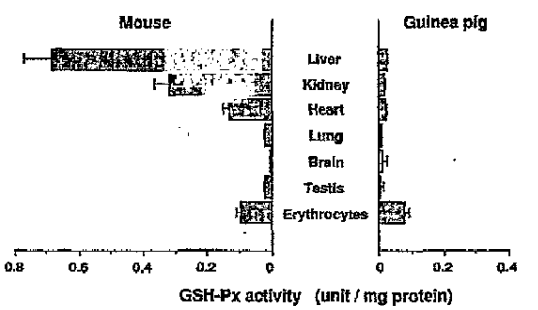

Fig. 1. GSH-Px activity in tissues of mice and guinea pigs. Selenium-dependent GSH-Px activity was determined with H_2O_2 as a substrate in all tissues except for the erythrocytes in which *tert*-butylhydroperoxide was used. One unit corresponds to 1 μmol NADPH oxidized/min.

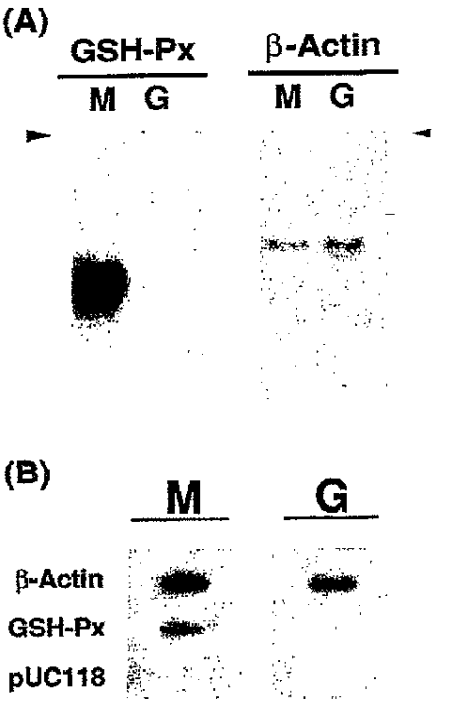

Fig. 2. Species difference in the expression of GSH-Px gene between mice and guinea pigs. (A) Northern blot analysis was performed using total RNA prepared from the liver of mice (M) and guinea pigs (G) probed with mouse GSH-Px cDNA and mouse β-actin cDNA fragments. Arrow heads indicate the origin (upper) in the electrophoresis and the positions of 28S (middle) and 18S (lower) rRNA. (B) Nuclear run-on assay was performed using the nuclei prepared from the liver of mice (M) and guinea pigs (G). Conditions for hybridization to GSH-Px and β-actin were the same as in the Northern blot analysis. pUC 118 plasmid DNA was used as a negative control.

Southern blot analysis of β-actin for DNAs obtained from mouse and guinea pig liver and confirmed that guinea pigs have homologous gene(s) to mouse β-actin in their genome (data not shown). The condition for hybridization to β-actin cDNA probe in Southern blot analysis was followed in the Northern blot analysis to quantitate the level of mRNA between the two species. In a previous study [7] we confirmed the condition for determining the mRNA level of GSH-Px of guinea pigs using mouse GSH-Px cDNA as a probe. The reaction conditions for β-actin were coincidentally the same as those for GSH-Px.

Fig. 2A shows the results of Northern blot analysis of GSH-Px and β-actin. As previously reported [7], mRNA of GSH-Px in the liver of guinea pigs was barely detectable. On the other hand, the level of β-actin mRNA did not significantly differ between mice and guinea pigs in the liver. Therefore, we used

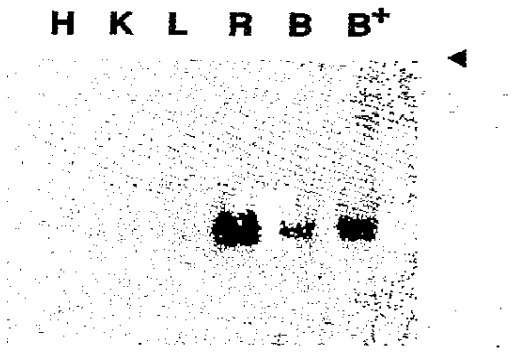

Fig. 3. Northern blot analysis of GSH-Px mRNA in guinea pigs. Total RNA was isolated from the heart (H), kidney (K), liver (L) and bone marrow cells (B) of non-treated guinea pigs. Guinea pigs were treated with phenylhydrazine for 5 days and total RNA was isolated from the reticulocytes (R) and bone marrow cells (B^+) on the third day after the last injection of phenylhydrazine. Arrow heads indicate the origin (upper) in the electrophoresis and the positions of 28S (middle) and 18S (lower) rRNA.

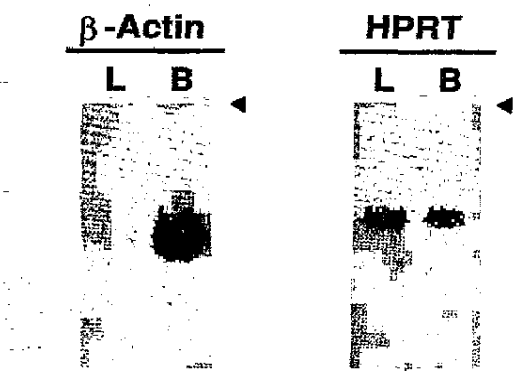

Fig. 4. Levels of mRNA of β-actin and HPRT in the liver and bone marrow cells of guinea pigs. Total RNA was isolated from the liver (L) and bone marrow cells (B) of guinea pigs. For the Northern blot analysis of HPRT, poly(A)$^+$ RNA was prepared from the total RNA using an oligo dT column. Mouse β-actin cDNA and mouse HPRT cDNA fragments were used as probes. Arrow heads indicate the origin (upper) in the electrophoresis and the positions of 28S (middle) and 18S (lower) rRNA.

β-actin as a control in the following nuclear run-on assay. As shown in Fig. 2B, the nuclear run-on assay revealed that only a faint signal indicative of GSH-Px gene transcription was detectable in the guinea pig liver. The intensity of signals of GSH-Px in the autoradiogram was evaluated by densitometry as a ratio to the signal of β-actin in each species. The ratios were 50% in mice and 5% in guinea pigs. Thus, the marked difference in hepatic GSH-Px activity between mice and guinea pigs may result primarily from the difference in the rate of GSH-Px gene transcription in the nuclei of the liver.

To determine whether the GSH-Px gene is expressed in the cells which differentiate into the erythrocytes in guinea pigs, Northern blot analysis was carried out. Fig. 3 clearly shows the difference in the level of GSH-Px mRNA among tissues and cells of guinea pigs. As previously reported [7], the signal of GSH-Px mRNA was hardly detectable in the liver, kidney and heart, while the reticulocytes showed a definite signal of GSH-Px mRNA. Bone marrow cells also showed a GSH-Px mRNA signal and a higher intensity of signal was observed in cells from phenylhydrazine-treated animals than in those from non-treated animals. In the phenylhydrazine-treated animals, the relative number of erythroblasts to total cells in the bone marrow increased by 20%.

The nuclear run-on assay was undertaken to determine whether the difference in the GSH-Px mRNA among tissues of guinea pigs actually reflects the difference in the rate of GSH-Px gene transcription. As shown in Fig. 4A, however, the levels of β-actin mRNA between the liver and bone marrow cells in guinea pigs differed considerably, indicating that β-actin is not a proper control for the nuclear run-on assay. Therefore, the level of HPRT mRNA was compared between the liver and bone marrow cells in guinea pigs since HPRT gene expression is also known to be stable [22]. Before Northern blot analysis, cross-hybridization of mouse HPRT cDNA to guinea pig gene was confirmed by Southern blot analysis (data not shown). Poly(A)$^+$ RNA instead of total RNA was used to determine the level of HPRT mRNA in guinea pig tissues since clear signals of HPRT mRNA could not be obtained by total RNA for reasons unknown. As shown in Fig. 4B, the difference in mRNA level of HPRT between the liver and bone marrow cells in guinea pigs was negligible.

Using HPRT as a control, a nuclear run-on assay was performed to examine the difference in the transcription rate of GSH-Px gene between the liver and bone marrow cells of guinea pigs. As shown in Fig. 5,

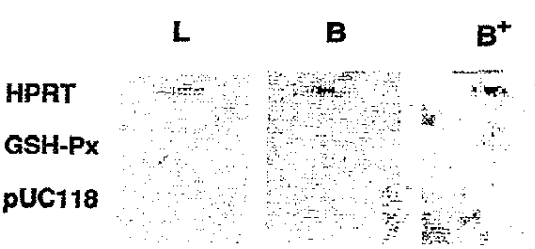

Fig. 5. Transcription rate of GSH-Px in the liver and bone marrow cells of guinea pigs. Nuclear run-on assay was performed using the nuclei prepared from the liver (L) and bone marrow cells (B) of non-treated guinea pigs, and the bone marrow cells (B^+) of phenylhydrazine-treated guinea pigs in the same way as described in Fig. 3. Mouse GSH-Px cDNA, mouse HPRT cDNA and pUC118 plasmid DNA were used as probes.

the signal indicating GSH-Px gene transcription in the liver of guinea pigs was barely detectable, which was the same as the results in Fig. 2. However, a definite signal was found in the bone marrow cells obtained from phenylhydrazine-treated animals. Densitometry of signals in autoradiograms revealed that the relative intensity of GSH-Px to HPRT were 5%, 10% and 30% in the liver, bone marrow cells from non-treated animals and those from phenylhydrazine-treated animals, respectively. These data indicated that the GSH-Px gene is transcribed in the erythroblasts or erythroid cells in the bone marrow of guinea pigs, and the difference in GSH-Px activity among tissues of guinea pigs may primarily be attributable to the difference in the rate of GSH-Px gene transcription, although involvement of other factors such as difference in the stability of mRNA among tissues cannot completely excluded.

Discussion

Guinea pigs have a characteristic tissue specificity of GSH-Px activity; very low activity in the major tissues such as the liver, kidney and heart, but higher activity in the erythrocytes than in the other tissues and comparable to that in mice or rats. The present study investigated the step in the gene expression responsible for the above-mentioned difference in GSH-Px activity in guinea pigs. The results obtained clearly indicated that the tissue specificity of GSH-Px activity in guinea pigs is due to the difference in the rate of GSH-Px gene transcription.

We have already demonstrated that the guinea pig has a gene highly homologous to mouse GSH-Px in its genome, presumably as a single copy [7]. Since mRNA of GSH-Px was hardly detectable in the liver, kidney and heart of guinea pigs, it has been suggested that the GSH-Px gene is faintly transcribed in these tissues of guinea pigs [7]. It appears to be reasonable, in general, to assume that the level of mRNA is principally regulated by the rate of transcription. In the case of GSH-Px, however, the mRNA level of GSH-Px in mice subjected to dietary Se deficiency was decreased without any change in the transcription rate of the GSH-Px gene as judged by the results of the nuclear run-on assay [11], suggesting post-transcriptional regulation of mRNA level by dietary Se. Since the UGA codon in the open reading frame of GSH-Px should be utilized for the incorporation of selenocysteine by an intricate mechanism [2–4], it is possible to presume that Se deficiency-induced changes in certain factors such as the conversion rate of suppressor tRNA charging serine to selenocysteyl tRNA may influence the stability of mRNA of GSH-Px. Although tissue Se concentrations in guinea pigs were not lower than those in mice or rats [7], the possibility cannot be excluded that the inability of Se utilization in the synthesis of GSH-Px in guinea pigs may lead to the rapid degradation of mRNA of GSH-Px. Therefore, to confirm the reason for the non-detectable level of GSH-Px mRNA in major tissues of guinea pigs, it is necessary to find out if the transcription rate of GSH-Px gene is very low or if post-transcriptional factor(s) responsible for stabilizing GSH-Px mRNA is lacking in this species.

The results of the nuclear run-on assay shown in Fig. 2 clearly demonstrated that the difference in the level of GSH-Px mRNA in the liver between mice and guinea pigs reflects the difference in the transcription rate of the GSH-Px gene. This may suggest that the factor(s) necessary for the transcription of GSH-Px gene may be lacking or inhibited in the liver of guinea pigs.

Evidence so far obtained for major tissues tends to imply that the GSH-Px gene itself in guinea pigs is inactive due to some defect and cannot be efficiently transcribed in any tissues of guinea pigs. However, the results of our recent study [8] and the data in Fig. 1 indicate that the GSH-Px gene in guinea pigs might be transcribed in the cells which differentiate into the erythrocytes. To examine this possibility, we performed Northern blot analysis using total RNA obtained from the reticulocytes of guinea pigs. Results shown in Fig. 3 suggest that the GSH-Px gene is transcribed in the erythroblasts or erythroid cells of guinea pigs. The nuclear run-on assay on bone marrow cells enriched with the erythroblasts by phenylhydrazine treatment has proved that GSH-Px gene is definitely transcribed in the erythroblasts in guinea pigs (Fig. 5).

As phenylhydrazine is an oxidant stressor [23], there remains a possibility that higher expression of GSH-Px gene in the bone marrow cells of phenylhydrazine-treated animals is merely a reflection of oxidant-responsive enhancement of GSH-Px gene expression. However, this possibility is unlikely since there is no alteration by phenylhydrazine treatment in the expression of the GSH-Px gene in the liver of guinea pigs (data not shown).

Results of this study collectively suggest that the GSH-Px gene in guinea pigs is not uniformly inactive in any tissues but actively transcribed in certain cells such as the erythroblasts or erythroid cells. Participation of *cis*- or *trans*-acting factor(s) in the regulation of this tissue-specific expression of GSH-Px gene in guinea pigs cannot be excluded. Whether these factors are lacking or inhibited in the major tissues of guinea pigs should be clarified in future studies. Chambers et al. [2] reported that the mouse GSH-Px gene has the consensus sequences as an Sp1 binding site but no 'TATA' and 'CAAT' sequences upstream of the initiation site for transcription like other house-keeping enzymes, which has also been confirmed in the rat GSH-Px gene [24]. So far, however, no factors regulat-

ing transcription of the GSH-Px gene have been characterized or identified. Thus, the guinea pig may serve as a good model for studying the factors regulating tissue-specific transcription of the GSH-Px gene.

It would be intriguing to find out why the GSH-Px gene is transcribed only in the cells differentiating into erythrocytes in guinea pigs. One possible reason might be the necessity of an antioxidant in the erythrocytes which convey oxygen and are therefore susceptible to oxidative stress. Interestingly, the compensatory role of catalase and glutathione *S*-transferase in the liver and kidney of guinea pigs has been suggested [8]. However, no such compensation for the removal of lipid hydroperoxides can be expected in the erythrocytes since non-Se GSH-Px activity of glutathione S-transferase is lacking [8]. In addition, it should also be taken into consideration that during the maturation process from the erythroid cells to erythrocytes, the organelles of these cells were decomposed in certain steps [25] accompanied by activation of a specific lipoxygenase [26]. This process may enhance the lipid peroxidation which can be scavenged only by GSH-Px in these cells. It is noteworthy that the GSH-Px gene was first identified as a highly expressed gene named ep19 in the erythroid cells [27]. The critical role of GSH-Px in the cells in the maturation process rather than in the erythrocytes may also be supported by the fact that the GSH-Px mRNA levels in the reticulocytes were markedly higher than those in the liver or kidney, although the activity of GSH-Px in the erythrocytes is not remarkably higher than in the other tissues of guinea pigs (Fig. 1).

Recently, several kinds of selenoenzymes other than GSH-Px have been identified in mammals; i.e., selenoprotein P [28], extracellular GSH-Px [29], phospholipid-hydroperoxide GSH-Px (PHGPX) [30] and type I iodothyronine 5′ deiodinase [31]. Since all of these selenoproteins contain Se as selenocysteine which is coded by UGA termination codon, the same mechanism for selenocysteine incorporation as that for GSH-Px may be the case of these selenoproteins. The guinea pig, as an unique animal in terms of the highly tissue-specific regulation of GSH-Px gene expression, may also be a good model for studying the regulation mechanism of the newly identified selenoproteins. Studies on other selenoproteins in guinea pigs are now under way in our laboratory.

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